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INVESTIGATIONS IN FISH CONTROL

51. Methods for Simultaneous Determination and Identification of MS-222 and Metabolites in Fish Tissues

52. Residues of MS-222, Benzocaine, and Their Metabolites in Striped Bass Following Anesthesia

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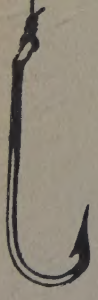

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METHODS FOR SIMULTANEOUS DETERMINATION AND IDENTIFICATION OF MS-222 AND METABOLITES IN FISH TISSUES

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ABSTRACT.--MS-222 (methanesulfonate of meta-aminobenzoic acid ethyl ester) is a primary aromatic amine commonly used to anesthetize fish. Like all primary aromatic amines, its diazonium salt reacts with N-1-naphthylethylenediamine dihydrochloride to form a wine-red azo dye with a maximum absorbance at 545 nm. Basic carbon tetrachloride extraction separates the azo dyes of MS-222 and its acid metabolite, m-aminobenzoic acid, and quantitative determination of each compound is made colorimetrically. By this method, recoveries of 82 to 110 percent for MS-222 and 84 to 117 percent for m-aminobenzoic acid were obtained from largemouth bass muscle and liver tissues spiked with 1 to 10 μg/g of each compound. Confirmation of MS-222 and m-aminobenzoic acid residues in fish tissue was made by thin-layer chromatography. Quantitative estimation and identification of each compound were possible from samples spiked with as little as 1 μg/g of MS-222 or m-aminobenzoic acid.

INTRODUCTION

MS-222 (methanesulfonate of meta-aminobenzoic acid ethyl ester) is a commonly used fish anesthetic. When any chemical or drug is used on a food commodity item, the U.S. Food and Drug Administration requires data on residual amounts of the drug and its metabolites left in the commodity after treatment. Previous methods of analysis for MS-222 residues in fish tissue did not identify m-aminobenzoic acid, a metabolite of MS-222. The method described by Walker and Schoettger (1967) included the residue of m-aminobenzoic acid, but the method did not separate it from MS-222 residues. The thin-layer chromatographic method of Allen, Luhning, and Harman (1970) only confirmed residues of MS-222 determined by the first method.

The analytical method described here will detect residues of MS-222 and its metabolites, namely, acetylated MS-222, m-aminobenzoic acid and m-acetylaminobenzoic acid. The thin-layer chromatographic procedures are done simultaneously with the analytical procedures, which save time and materials.

METHODS AND MATERIALS

Reagents and apparatus

Reference to a company or product does not imply recommendation to the exclusion of others that may be suitable. Reagents and apparatus used were as follows:

1. Carbon tetrachloride, reagent grade.
2. Trichloroacetic acid: Dissolve 60 g crystalline TCA in distilled water. Transfer to a 100-ml volumetric flask and bring to volume with distilled water. Dilute 50 ml to 200 ml to obtain 15-percent TCA, and dilute 5 ml of the original stock to 100 ml to obtain 3-percent TCA.

3. 0.2-percent sodium nitrite: Transfer 0.2 g $NaNO_2$ into a 100-ml volumetric flask, dissolve, and bring to volume with distilled water. Make fresh daily.

4. 0.5-percent ammonium sulfamate: Transfer 0.5 g $NH_4SO_3NH_2$ into a 100-ml volumetric flask and bring to volume with distilled water.

5. 0.1-percent N-1-naphthylethylenediamine dihydrochloride: Transfer 0.1 g $C_{12}H_{14}N_2$-2HCl into a 100-ml volumetric flask, dissolve, and bring to volume with distilled water. Refrigerate and make up fresh weekly.

6. 4N hydrochloric acid: Dilute 40 ml 10N HCl to 100 ml with distilled water.

7. 10-percent hydrochloric acid (V/V).

8. Concentrated ammonium hydroxide.

9. Standard solutions: Weigh 0.01 g of MS-222 and quantitatively transfer to a 100-ml volumetric flask, add 20 ml of 15-percent TCA and bring to volume with distilled water. One ml of this stock solution diluted to 100 ml with 3-percent TCA will make a 1 μg/ml solution. Make fresh daily. Standards of m-aminobenzoic acid are made up weekly in like manner.

10. Ethyl acetate, reagent grade.

11. Iso-butanol, reagent grade.

12. Developing solutions: The first solution contains 180 ml ethyl ether, 10 ml acetone, and 10 ml concentrated glacial acetic acid. The second solution contains 120 ml chloroform, 38 ml ethyl alcohol, 38 ml methanol, and 4 ml concentrated ammonium hydroxide. All solvents are reagent grade or better.

13. Waring blendor.

14. Tissue homogenizer, Virtis "45".

15. Orbital centrifuge.

16. Chromatography tank 10.2 by 20.3 by 22.9 cm, lined with absorbent paper.

17. Silica gel thin-layer plates, Eastman chromagram sheets without flourescent dye, 20 by 20 cm.

18. Chromatography spray bottle.

19. Micropipettes, 1, 5, 10, and 25 μl.

20. Centrifuge tubes, 15 and 40 ml.

21. Spectrophotometer cuvettes, round, 13 by 100 mm.

22. Spectrophotometer, Bausch and Lomb "Spectronic 20".

23. Whatman #40 filter paper, 9.0 cm diameter.

24. Funnels, 5 cm diameter.

Tissue collection and treatment

Blood samples are taken by caudal puncture (Steucke and Schoettger, 1967) with a heparinized syringe fitted with a 20-gauge needle. Place 1.0 ml of blood in a 40-ml graduated centrifuge tube, and add 15 ml of distilled water and 4 ml of 15-percent TCA. Mix thoroughly and centrifuge at 2,500 RPM for 30 minutes. Filter the supernatant through #40 Whatman filter paper, and the sample is ready for analysis. If a 1.0-ml sample of blood cannot be obtained, use the amount obtained and proportional amounts of water and TCA.

Fillet each fish after the other tissues have been removed, and homogenize the entire fillet in a Waring blendor to obtain a homogeneous sample (Luhning and Harman, 1971). Weigh out 1.0 g of homogenized tissue in a tared homogenizing flask, add 10 ml of distilled water and homogenize for 1 minute. Quantitatively transfer to a 40-ml graduated centrifuge tube and bring to a volume of 16 ml with distilled water. Then add 4 ml of 15-percent TCA to coagulate the protein, mix thoroughly, and centrifuge at 2,500 RPM for 20 minutes. Filter the supernatant through #40 Whatman filter paper, and the sample is ready for analysis. Kidney, liver, and brain are dissected from the fish and prepared the same as muscle tissue, except that these samples are not homogenized before weighing out a 1.0-g sample.

Analytical procedures

The first seven steps are basically the same as those described by Walker and Shoettger (1967). The procedure is as follows:

1. Pipette 5 ml of 3-percent TCA into a clean 50-ml Erlenmeyer flask for a reagent blank.

2. Pipette 5 ml of 1-μg/ml standard of MS-222 and m-aminobenzoic acid into Erlenmeyer flasks. Also pipette 5 ml of mixed MS-222 and acid standards, each at a 1-μg/ml concentration, into an Erlenmeyer flask.

3. Pipette 5 ml of filtrate from each sample into separate 50-ml Erlenmeyer flasks.

4. Add 0.5 ml of 0.2-percent sodium nitrite to each flask, swirl, and let stand for 15 minutes. Keep samples at room temperature and out of direct sunlight.

5. Add 0.5 ml of 0.5-percent ammonium sulfamate to each flask, swirl, and let stand for 3 minutes.

6. Add 0.5 ml of 0.1-percent N-1-naphthyl-ethylenediamine dihydrochloride to each flask, swirl vigorously, and let stand for 10 minutes.

7. Pour samples into a clean cuvette. Zero spectrophotometer at 100-percent transmittance using the reagent blank. Read percent transmittance of each sample in the spectrophotometer at 545 nm.

8. Transfer the azo dyestuff to a labeled 15-ml centrifuge tube containing 0.5 ml of 7.5 M ammonium hydroxide and 2 ml of carbon tetrachloride. Shake vigorously, and centrifuge at 1,500 RPM for 3 minutes.

9. Rinse each cuvette with distilled water and shake dry. Be sure to keep cuvettes in order. Add 1 ml of 60-percent TCA to each cuvette.

10. After centrifugation (step 8), transfer 5 ml of the aqueous layer back into the original cuvette and mix gently.

11. Zero the spectrophotometer at 100-percent transmittance using the extracted reagent blank, and again read percent transmittance of each sample with a spectrophotometer at 545 nm.

12. Convert all percent transmittance readings to absorbance, and calculate μg/g residues as follows:

$$\mu g/g \text{ free MS-222} = \frac{S_1 \times \frac{A_2}{A_1} - S_2}{M_1 \times \frac{A_2}{A_1} + M_2} \times 20$$

$$\mu g/g \text{ free m-aminobenzoic acid} = \frac{S_2}{A_2} \times 20$$

S_1 = absorbance of sample before extraction.

S_2 = absorbance of sample after extraction.

M_1 = absorbance of mixed standard (1 μg/ml MS-222 and 1 μg/ml m-aminobenzoic acid) before extraction.

M_2 = absorbance of mixed standard after extraction.

A_1 = absorbance of m-aminobenzoic acid standard (1-μg/ml) before extraction.

A_2 = absorbance of m-aminobenzoic acid after extraction.

13. Possible acetylated derivatives can be analyzed as follows:

A. Place 5 ml of filtrate from each sample into separate 15-ml graduated centrifuge tubes and add 0.5 ml of 4N HCl.

B. Place tubes in a boiling water bath for 1 hour, cool and adjust volume to 5 ml with distilled water.

C. Transfer to a 50-ml Erlenmeyer flask and analyze as before, starting with step 4.

D. The amount of acetylated residues is obtained by subtracting the concentration of the first analysis (free residues) from the concentration of the second analysis.

14. For the identification and confirmation of MS-222 and m-aminobenzoic acid residues by thin-layer chromatography, proceed as follows:

A. React another 5 ml of filtrate from each sample and standards with the Bratton-Marshall reagents (steps 1 through 6).

B. Transfer the azo dyestuff to a stoppered test tube containing 1 ml of iso-butanol-ethyl acetate (50/50, V/V). Shake vigorously, and let stand for 30 minutes in a dark place.

C. Mark a spotting line 2.5 cm and a solvent-front line 12.5 cm from the bottom of a 20 by 20 cm silica gel thin-layer plate.

D. With a calibrated micropipette, spot enough iso-butanol-ethyl acetate on spotting line to give a visible, colored spot for each sample. On the same plate, spot 1, 2, and 5 μl of the mixed standard (1- μg/ml concentration of each).

E. Thirty minutes before developing the plate, mix 180 ml of ethyl ether, 10 ml of acetone, and 10 ml of concentrated acetic acid (90:5:5) and pour into a chromatography tank lined with absorbent paper. Place the plate in the tank and allow the solvent to rise to the top of the plate, then remove and dry under an electric hair dryer in a fume hood.

F. Develop the plate again in 120 ml of chloroform, 38 ml of ethanol, 38 ml of methanol, and 4 ml of concentrated ammonium hydroxide (60:19:19:2). Allow the solvent front to migrate up to the previously marked line (12.5 cm from the bottom), then remove the plate, dry, and spray with 10-percent HCl under a fume hood.

G. Compare the R_f values of the standards to those of the samples. In general, the R_f values will be 0.83 for MS-222 and 0.18 for m-aminobenzoic acid. An estimate of the concentration present in the samples can be made by comparing the color intensity and size of the sample spot to that of a spot obtained from a known amount of standard.

A flow chart has been prepared to diagram the sequence of the above procedures (fig. 1). The chemical structures of MS-222 and its metabolites are illustrated in figure 2.

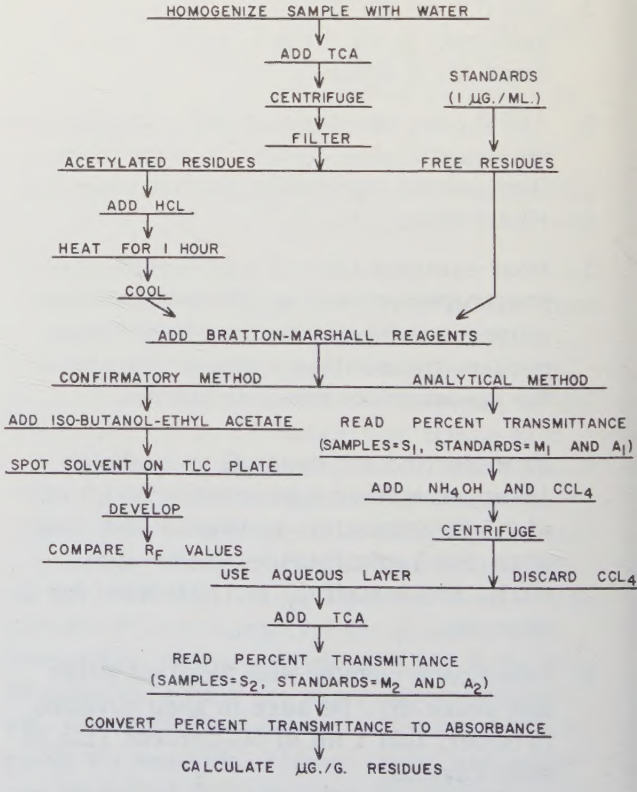
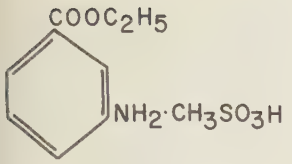
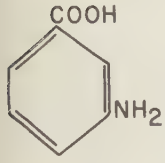


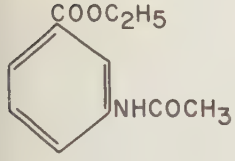
Figure 1.--Flow chart of procedures for the determination and identification of MS-222 and metabolites.



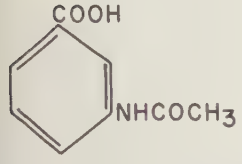
Methanesulfonate of m-aminobenzoic acid ethyl ester (MS-222)



m-Aminobenzoic acid



m-Acetylaminobenzoic acid ethyl ester



m-Acetylaminobenzoic acid

Figure 2.--Chemical structures of MS-222 and metabolites.

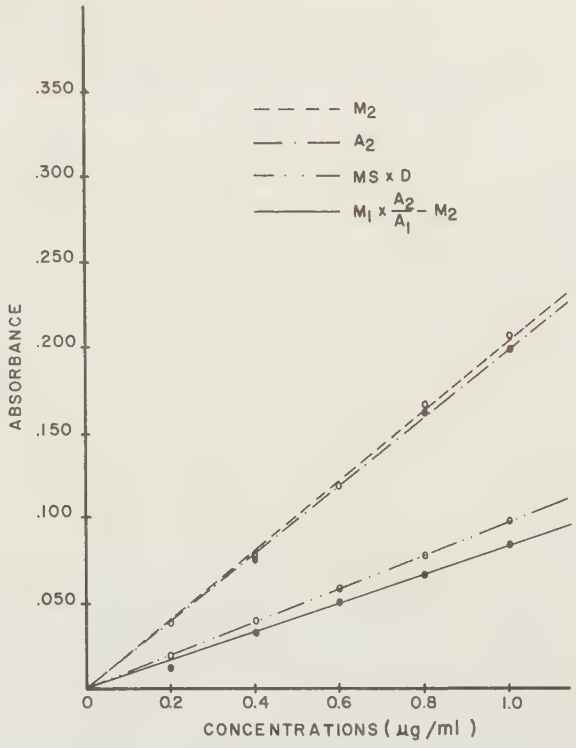


Figure 3.--Absorbance curves at 545 nm for: (A_2) standards of m-aminobenzoic acid extracted with carbon tetrachloride: (M_2) standards of MS-222 and m-aminobenzoic acid mixed and extracted with carbon tetrachloride; ($M_1 \times \frac{A_2}{A_1} - M_2$) the calculated absorbance of MS-222 after extracting the acid from the mixed standards; and (MS X D) standard curve of MS-222 multiplied by the known dilution factor resulting from the extraction of the acid.

RESULTS

The efficiency of the extraction procedure was evaluated by analyzing a series of pure and mixed standards of MS-222 and m-aminobenzoic acid before and after extraction with basic carbon tetrachloride (fig. 3). The concentration of the standard did not influence the amount extracted, for the regression of absorbance values was relatively linear with increasing concentration of the standards. The absorbance value for the pure m-aminobenzoic acid standard before extraction, divided by the absorbance value after extraction (A_2/A_1) does not equal the known dilution factor of the procedure (D). The difference is attributed to a small loss of material left in the cuvette after transferring the material to a centrifuge tube. Thus, in figure 3, the slope of the absorbance curve for MS-222 obtained from the mixed standards ($M_1 \times A_2/A_1 - M_2$) is slightly different from one calculated by the known dilution factor (MS X D).

When a fresh standard of MS-222 is diazotized and extracted with basic carbon tetrachloride, the aqueous fraction has zero absorbance. Thus, the amount of MS-222 present in a sample has to be calculated from the information obtained by extracting a 1-µg/ml standard of acid and the absorbance values of a mixed 1-µg/ml standard of MS-222 and m-aminobenzoic acid before and after extraction.

The amount of recoverable MS-222 and m-aminobenzoic acid was determined by spiking samples of largemouth bass (Micropterus salmoides) and liver tissue with 1 to 10 µg/g amounts of the two compounds (table 1). Recovery studies were done only on muscle and liver tissues because interfering substances

Table 1.--Recovery of MS-222 and m-aminobenzoic acid spiked into 1-g samples of largemouth bass muscle and liver tissue as determined by the colorimetric method and confirmed by the thin-layer chromatographic method

Tissue	Number of analyses	Concentration added (μg)		Concentration recovered (μg)		Percent recovery		Confirmed by TLC	
		MS-222	Acid	MS-222	Acid	MS-222	Acid	MS-222	Acid
Muscle	3	1.0	0.0	0.827	--	82.7	--	Yes	No
	3	5.0	0.0	4.740	--	94.8	--	Yes	No
	3	7.5	0.0	6.750	--	90.0	--	Yes	No
	3	10.0	0.0	10.680	--	106.8	--	Yes	No
	3	0.0	1.0	--	0.860	--	86.0	No	Yes
	3	0.0	5.0	--	4.970	--	99.6	No	Yes
	3	0.0	7.5	--	7.470	--	99.6	No	Yes
	3	0.0	10.0	--	9.950	--	99.5	No	Yes
	3	1.0	1.0	0.870	0.910	87.0	91.0	Yes	Yes
	3	2.5	7.5	2.730	7.340	109.2	97.9	Yes	Yes
	3	5.0	5.0	4.790	4.870	95.8	97.4	Yes	Yes
	3	7.5	2.5	7.720	2.180	102.9	87.2	Yes	Yes
Liver	1	5.0	0.0	4.880	--	97.6	--	Yes	No
	1	10.0	0.0	10.230	--	102.3	--	Yes	No
	1	0.0	5.0	--	4.780	--	95.6	No	Yes
	1	0.0	10.0	--	10.500	--	105.0	No	Yes
	1	2.5	7.5	2.650	8.770	106.0	116.9	Yes	Yes
	1	7.5	2.5	8.270	2.100	110.3	84.0	Yes	Yes

are most prevalent in liver and least prevalent in muscle tissue. Recoveries ranged from 82.7 to 110.3 percent for MS-222 and from 84.0 to 116.9 percent for m-aminobenzoic acid.

Diazotization of compounds mentioned in this study was accomplished by modification of the Bratton and Marshall (1939) procedure described by Walker and Schoettger (1967). The reaction of primary aromatic amines with nitrous acid yields a diazonium salt, which will couple with certain aromatic amines to yield strongly colored azo compounds. Naturally occurring primary aromatic amines in certain fish tissues, especially liver, react with the Bratton-Marshall reagents and cause serious quantitative errors in the determination of MS-222 and m-aminobenzoic acid residues. Thus, background levels of these amines are determined in control samples and substracted from levels obtained in treated samples. This is an accepted procedure in any analytical method by spectrophotometry.

Another standard procedure should be a confirmatory method of analysis. The thin-layer chromatographic method described previously will separate azo dyestuffs of naturally occurring primary aromatic amines from MS-222 and m-aminobenzoic acid. The following R_f values were obtained: 0.83 for the azo dyestuff of MS-222, 0.70 for m-aminophenol, 0.34 for sulfanilic acid, 0.26 for p-aminobenzoic acid, 0.18 for m-aminobenzoic acid, and 0.00 for liver extract. The comparison of R_f values must be made on the same thin-layer plate since these values may vary between determinations.

The minimum amount of azo dyestuff from a MS-222 standard that can be visualized on a

thin-layer plate was found to be 5 ng. The maximum amount of azo dyestuff from muscle extract that can be spotted on a thin-layer plate is 100 µl, which is equivalent to 25 mg of tissue. Thus, the sensitivity of the method is 0.2 ng/mg. Quantitative estimations of residues in samples can be accomplished by spotting a series of MS-222 and m-aminobenzoic acid standards in the range of 5 to 25 ng amounts on the same plate along with the samples.

DISCUSSION

The analytical and confirmatory methods described here will effectively determine and positively confirm which metabolites of MS-222 are present. Different species of fish seem to eliminate residues of MS-222 by various means of excretion and deactivation, which in turn will influence the amount of metabolites present in the muscle (table 2).

Excellent recoveries of MS-222 and m-aminobenzoic acid were obtained from spiked samples of muscle tissue. Recoveries were higher from spiked liver samples because of inconsistent background interference from naturally occurring primary aromatic amines, which amounted to a mean of 89 percent transmittance in the control liver samples after extraction. By thin-layer chromatography, the control liver samples contained no spot with the same R_f value as MS-222 or m-aminobenzoic acid.

The accuracy of the analytical method is governed by the tissue being analyzed and by the techniques of extraction and dilution. The dilution factor attributed to extraction

Table 2.--Average concentrations of MS-222, m-aminobenzoic acid, and their N-acetyl derivatives in muscle tissue of four species of fish after a 15-minute exposure to a 100-mg/l aqueous solution of MS-222 at 18° C.

	Number of fish	Mean concentration of residues by colorimetric method				Free residues confirmed by TLC	
		Free MS-222 (µg/g)	Acetylated MS-222 (µg/g)	Free acid (µg/g)	Acetylated acid (µg/g)	MS-222	Acid
Channel catfish (Ictalurus punctatus)	5	67.9 [1](0.7)	4.5	3.4 (0.5)	1.7	Yes	Yes
Striped bass (Morone saxatilis)	5	23.0 (0.5)	0.0	14.7 (1.2)	3.3	Yes	Yes
Bluegill (Lepomis macrochirus)	5	23.8 (1.0)	0.6	0.0 (0.0)	0.0	Yes	No
Largemouth bass (Micropterus salmoides)	5	34.4 (1.9)	0.0	1.4 (0.0)	0.7	Yes	Yes

[1] Residues after 6 hours recovery in fresh water at 18° C; not analyzed by TLC.

($A_2/A_1 = 0.725$) as determined by the extraction of a 1-μg/ml standard of m-aminobenzoic acid was quite consistent. Particular care must be taken to remove all gas bubbles that form on the sides of the cuvettes. Generally, these bubbles will float to the top when the sides of the cuvette are tapped lightly with a soft object. The maximum absorbance for the azo dyestuff of m-aminobenzoic acid is identical to that of MS-222, which is 545 nm.

CONCLUSIONS

1. The colorimetric method presented will effectively determine MS-222 and m-aminobenzoic acid residues in fish tissues.
2. Recovery of the compounds from spiked samples of muscle and liver tissues ranged from 82.7 to 110.3 for MS-222 and from 84.0 to 116.9 percent for m-aminobenzoic acid.
3. The thin-layer chromatographic method presented will positively identify and separate MS-222 and m-aminobenzoic acid residues in fish tissues.
4. The R_f values for the azo dyestuff of MS-222 and m-aminobenzoic acid are near 0.83 and 0.18, respectively.
5. Quantitative estimation and identification by the thin-layer chromatographic method were possible from samples spiked with as little as 1 μg/g of MS-222 or m-aminobenzoic acid.

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52. Residues of MS-222, Benzocaine, and Their Metabolites in Striped Bass Following Anesthesia

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Fish and Wildlife Service
Bureau of Sport Fisheries and Wildlife

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RESIDUES OF MS-222, BENZOCAINE, AND THEIR METABOLITES IN STRIPED BASS FOLLOWING ANESTHESIA

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ABSTRACT.--Striped bass (Morone saxatilis) anesthetized in a 100-mg/l solution of MS-222 at 17.5° C contained an average of 57.9 μg/g of MS-222 and 23.3 μg/g of m-aminobenzoic acid residues in the muscle tissue immediately after a 30-minute exposure to the drug (0-hour withdrawal samples). After this tissue was homogenized and stored in a refrigerator for 1 week at 1.7° C, residues were 100 percent m-aminobenzoic acid. Fish anesthetized with benzocaine and treated in like manner still contained residues of benzocaine and a small amount of p-aminobenzoic acid (3.4 percent) after storage. The ester and acid residues of both anesthetics decreased steadily with length of recovery time. The residues were measured by a modified Bratton-Marshall colorimetric method and confirmed by thin-layer chromatography.

INTRODUCTION

MS-222 (methanesulfonate of meta-aminobenzoic acid ethyl ester) is an effective and widely used fish anesthetic (Schoettger, 1967; Schoettger and Julin, 1967; Schoettger, Walker, Marking, and Julin, 1967). Residues of this drug occur in various tissues of anesthetized fish, but decline to background levels within 9-24 hours after fish are placed into fresh water (Walker and Schoettger, 1967a). Other than the fact that residues do disappear, little is known about the metabolic fate of MS-222. In striped bass muscle tissue analyzed by the modified method of Walker and Schoettger (1967b), MS-222 residues were found in 0-hour through 8-hour withdrawal samples. When these same samples were analyzed by the thin-layer chromatographic method (Allen, Luhning, and Harman, 1970), only the 0-hour withdrawal samples contained MS-222 residues. These results implied that the former method did not distinguish between MS-222 and some metabolite. The metabolite was identified as m-aminobenzoic acid and was analyzed by the method of Luhning (1973).

The objective of this study was to determine the amount of MS-222 and m-aminobenzoic acid residue in muscle tissue of striped bass anesthetized with MS-222. Analyses of benzocaine, a structural analogue of MS-222, and p-aminobenzoic acid residues in muscle tissue of striped bass, bluegill, and largemouth bass anesthetized with benzocaine were included for comparative purposes.

METHODS AND MATERIALS

Fish

Striped bass (Morone saxatilis), ranging from 12.7 to 20.3 cm long, were obtained from the National Fish Hatcheries at Edenton, N.C., and Welaka, Fla. Bluegill (Lepomis macrochirus), ranging from 16.5 to 21.6 cm long, and largemouth bass (Micropterus salmoides), ranging from 16.5 to 25.4 cm long, were obtained from the National Fish Hatchery at Marion, Ala. All fish were held in limed, spring water at the Warm Springs Laboratory and were maintained according to the methods of Hunn, Schoettger, and Whealdon (1968).

Anesthetization of fish

I used technical-grade (99.4-percent) MS-222, the methane sulfonate of m-aminobenzoic acid ethyl ester, marketed for experimental purposes by Ayerst Laboratories, Inc., New York, N.Y., under the trade name of FINQUEL(R). The m- and p-aminobenzoic acid and p-aminobenzoic acid ethyl ester were purchased from Eastman Kodak Company.[1]

Striped bass, bluegill, and largemouth bass were anesthetized for 30 minutes in a 100-mg/l aqueous solution of MS-222 buffered to pH 7.0 with sodium bicarbonate. Also, these three species of fish were anesthetized for 15 minutes in a 63.216-mg/l aqueous solution of benzocaine (63.216 mg/l para-aminobenzoic acid ethyl ester is equal on a mole basis to 100 mg/l methanesulfonate salt of meta-aminobenzoic acid ethyl ester) buffered to pH 6.5 with sodium bicarbonate. All fish were exposed to 75 l of anesthetic solution at a temperature of $18 \pm 0.5^{\circ}$ C.

Benzocaine is only slightly soluble in water. To obtain the concentration of anesthetic equivalent to that of MS-222, the benzocaine was dissolved in ethanol containing a small amount of methanesulfonic acid. Thus, 4.7412 g of benzocaine dissolved in 50 ml of ethanol were added to 75 l of limed, spring water to obtain a concentration of anesthetic equivalent to 100 mg/l of MS-222.

After the specified exposure time, the fish were withdrawn from the anesthetic solution, and placed in fresh, flowing water for recovery. This action marked the beginning of withdrawal time. Fish killed immediately after withdrawal from the anesthetic solution were labeled as 0-hour withdrawal samples. Five fish were used for residue analyses at each withdawal interval.

Analyses of samples

The head, scales, fins, and viscera were removed from each fish, and the remaining tissues were homogenized in a Waring blendor. One-gram samples of these homogenates were analyzed for residues of MS-222, benzocaine, and their acid metabolites on the same day the fish were killed. The remainder of each homogenate was stored in a refrigerator at 1.7° C, and a sample was analyzed 1 week later. One-gram samples of liver and 1 ml of blood from some fish also were analyzed on the same day the fish were killed.

Samples of homogenized tissue were initially analyzed by the colorimetric method described by Walker and Schoettger (1967b). In addition to this method, the azo dyestuff of each sample was partitioned with basic carbon tetrachloride, centrifuged, and the aqueous layer separated, and made acidic (Luhning, 1973). This aqueous fraction contained only the azo dyestuff of the acid (m- or p-aminobenzoic acid).

Standard solutions of mixed ester and acid at a concentration of 1 μg/ml each, also were partitioned. The amounts of ester and acid present in the samples were calculated on a ratio basis with standards of the ester and acid partitioned in the same manner as the samples.

RESULTS

MS-222 treated fish

Twenty striped bass were anesthetized for 30 minutes in a 100-mg/l solution of MS-222 at 17.5° C. Deep anesthesia in striped bass occurred about the same time as it did in the largest specimens among the largemouth bass. No mortalities occurred with bluegill or largemouth bass, whereas one striped bass died during the 24-hour recovery period.

Striped bass rapidly hydrolyzed the m-aminobenzoic acid ethyl ester to m-aminobenzoic acid during exposure and withdrawal (table 1). During the first 4 hours of recovery, residues of m-aminobenzoic acid dissipated from the muscle tissue at a slower rate than did the MS-222 residues. For comparison, five bluegill and five largemouth bass anesthetized and analyzed in the same way as the striped bass contained a small percentage of total residue as acid at the 1-hour withdrawal interval.

[1] Reference to a company or product does not imply recommendation to the exclusion of others that may be suitable.

Table 1.--Average concentrations of MS-222 and m-aminobenzoic acid residues in muscle tissue of five striped bass, bluegill, and largemouth bass following deep anesthesia in a 100-mg/l aqueous solution of MS-222 buffered to pH 7.0 at 17.5° C

Species	Exposure time (minutes)	Withdrawal time (hours)	Free MS-222 residues (μg/g)		Free m-aminobenzoic acid residues (μg/g)	
			Mean± $s_{\bar{x}}$ [1]	Range	Mean± $s_{\bar{x}}$	Range
Striped bass[2]	0	0	0.0	--	0.3± 0.09	0.0- 0.5
	30	0	57.9± 5.59	36.1-66.5	23.3± 2.63	15.3-30.6
		1	6.5± 1.06	2.6- 8.9	16.5± 1.94	11.1-21.1
		4	0.8± 0.23	0.2- 1.5	7.5± 0.79	5.7-10.0
		24	[3]0.5± 0.16	0.2- 0.8	0.2± 0.06	0.0- 0.2
Bluegill[4]	30	1	4.8± 0.27	3.7- 5.2	0.5± 0.13	0.2- 0.9
Largemouth bass[5]	30	1	8.1± 0.61	5.8- 9.5	0.3± 0.10	0.0- 0.6

[1] Standard error of the mean for five fish.
[2] Average length of 15.2 cm and average weight of 46.9 g.
[3] Mean of four fish.
[4] Average length of 20.1 cm and average weight of 179.0 g.
[5] Average length of 20.3 cm and average weight of 154.0 g.

A limited number of striped bass were available to test the rate of hydrolysis during a 50-minute exposure to a 100-mg/l aqueous solution of MS-222 and at intervals during recovery from the drug in fresh, flowing water (table 2). The amount of residue accumulated after 30 minutes of exposure and no recovery time was 44.6 μg/g, of which 49.6 percent was free MS-222. This amount is not in agreement with the data found in tables 1 and 4. This discernible difference could have been due to fish size, number of fish analyzed, temperature difference, source of fish difference, or experimental error.

The added information gained from this test was the fact that m-aminobenzoic acid residues continued to increase during 50 minutes of anesthesia and MS-222 residues started to decrease after 30 minutes of anesthesia (fig. 1). Analyses of acetylated MS-222 and m-aminobenzoic acid also were performed on these samples, and only a small amount of the residue was found to be acetylated, mostly as m-acetylaminobenzoic acid (table 2).

Hydrolysis of MS-222 also occurred in homogenized striped bass tissue during storage (table 3). Samples of homogenized muscle tissue from striped bass, bluegill, and largemouth bass were previously analyzed for MS-222 and m-aminobenzoic acid residues (table 1). Additional samples were analyzed after storage for 1 week at 1.7° C. The 0-hour withdrawal samples of striped bass contained an average of 51.6 μg/g acid residues, but no residues of MS-222 could be detected at the end of the storage period. A reduction in the MS-222 concentration occurred during storage of

Table 2.--Residues of MS-222, m-aminobenzoic acid and their N-acetyl derivatives in muscle tissue of striped bass during different times of exposure to and recovery from a 100-mg/l aqueous solution of MS-222 buffered to pH 7.0 at 18.5° C

Exposure time (minutes)	Withdrawal time (minutes)	Average free[1] residues (µg/g)		Average acetylated[2] residues (µg/g)		Total residues (µg/g)	Percent free MS-222
		MS-222	Acid	MS-222	Acid		
15	0	19.0	11.9	0.0	3.3	34.2	55.6
30	0	22.1	19.4	0.0	3.1	44.6	49.6
50	0	15.8	24.3	0.0	2.5	42.6	37.1
30	15	13.2	17.8	0.0	2.2	33.2	39.8
30	30	12.3	11.6	0.0	2.0	25.9	47.5
30	45	7.8	11.6	0.5	1.5	21.4	36.4
30	60	[3]5.6	9.4	0.0	1.6	16.6	33.7

[1] Average of two fish having an average length and weight of 17.8 cm and 67.6 g., respectively.

[2] Obtained by subtracting the free residue and background concentration of control (untreated) fish from the amount measured in the samples after acid hydrolysis and extraction.

[3] Average of two analyses from one fish.

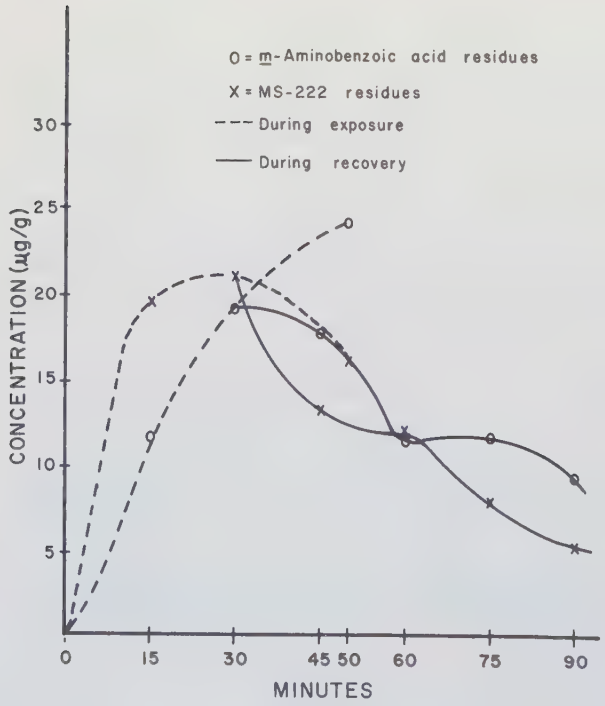

Figure 1.--Uptake and hydrolysis of MS-222 residues in striped bass muscle tissue during different lengths of exposure to a 100-mg/l solution of MS-222 buffered to pH 7.0 at 18.5° C, and elimination of MS-222 and m-aminobenzoic acid residues during 1 hour of recovery time in fresh water.

Table 3.--Residues of free MS-222, benzocaine, and their acid metabolites in homogenized muscle tissue of three species of treated fish stored for 1 week at 1.7° ± 0.2° C

Species	Withdrawal time (hours)	MS-222 treated[1]		Benzocaine treated[2]	
		MS-222 (μg/g)	m-aminobenzoic acid (μg/g)	Benzocaine (μg/g)	p-aminobenzoic acid (μg/g)
		Mean ± $s_{\bar{x}}$[3]	Mean ± $s_{\bar{x}}$	Mean ± $s_{\bar{x}}$	Mean ± $s_{\bar{x}}$
Striped bass	0	0.0	51.6 ± 5.73	25.8 ± 1.83	0.9 ± 0.10
	1	0.1 ± 0.07	16.5 ± 2.44	3.3 ± 0.19	0.2 ± 0.10
	4	0.5 ± 0.17	5.8 ± 0.81	0.6 ± 0.05	0.1 ± 0.04
Bluegill	1	3.8 ± 0.61 [4](0.0)	0.6 ± 0.08 (0.6)	1.5 ± 0.50 (0.0)	0.0 (0.6)
Largemouth bass	1	5.0 ± 0.99 (0.0)	0.4 ± 0.02 (0.6)	2.1 ± 0.32 (0.0)	0.06 ± 0.04 (0.4)

[1] Anesthetized for 30 minutes in a 100-mg/l solution of MS-222 buffered to pH 7.0 at 17.5° C.

[2] Anesthetized for 15 minutes in a 63.216-mg/l solution of benzocaine (equivalent to 100-mg/l of MS-222) buffered to pH 6.5 at 17.8° C.

[3] Standard error of the mean for five samples.

[4] Acetylated fraction obtained by subtracting the free and background concentrations of control (untreated) fish from the amount measured in the samples after acid hydrolysis and extraction.

homogenized muscle tissue from bluegill and largemouth bass, but no significant change occurred in the acid concentration (tables 1 and 3).

In another test using 0-hour withdrawal samples of striped bass tissue, the temperature at which samples were stored greatly influenced the rate at which MS-222 residues were hydrolyzed to the acid (tables 4 and 5). Homogenized tissue stored at -12.2° C for 3 weeks contained an average of 21.7 μg/g MS-222 residues. However, after this tissue was stored for an additional 8 days at 4.4° C, it contained no residues of MS-222.

Residues of MS-222 and m-aminobenzoic acid were confirmed in all the 0- and 1-hour withdrawal samples of striped bass by the thin-layer chromatographic method described by Luhning (1973).

Benzocaine treated fish

Striped bass, bluegill, and largemouth bass were anesthetized in a 63.216-mg/l aqueous solution of benzocaine for 15 minutes at 17.8° C. Deep anesthesia occurred in about the same time as it did when MS-222 was used. No mortalities of largemouth bass or bluegill occurred before the withdrawal time of 1 hour, but one striped bass died during the 24-hour recovery period.

Benzocaine residues comprised from 93.5 to 100 percent of the total residues found in these three species of fish (table 6). The mean residues of p-aminobenzoic acid in striped bass and largemouth bass were about the same concentration at the 1-hour withdrawal interval, whereas residues of m-aminobenzoic acid differed greatly between these two species at the same withdrawal interval (tables 1 and 6).

Table 4.--Residues of MS-222 and m-aminobenzoic acid in 0-hour withdrawal samples of striped bass muscle tissue during storage at -12.2° C

Storage time in weeks	Average µg/g free residues[1]				Percent MS-222
	MS-222		m-aminobenzoic acid		
	Mean	Range	Mean	Range	
0	35.4	30.4-44.9	6.9	5.6- 8.0	83.7
1	28.4	22.3-36.8	11.2	10.0-12.2	71.7
2	24.5	15.9-31.9	12.1	10.0-14.0	66.9
3	21.7	12.3-31.9	12.7	10.0-15.3	62.8
4	18.2	9.9-26.4	13.2	11.7-16.0	58.0
5	16.3	8.1-20.9	14.3	12.6-16.9	53.3
6	15.3	6.9-20.3	15.7	14.1-17.9	49.4
8	15.0	6.8-20.8	16.0	14.1-17.9	48.4

[1] Average of three fish, which were exposed for 30 minutes in a 100-mg/l aqueous solution of MS-222 at 20° C.

Table 5.--Residues of MS-222 and m-aminobenzoic acid in 0-hour withdrawal samples of striped bass muscle tissue kept at -12.2° C for 3 weeks, then analyzed during storage at 4.4° C

Storage time in days	Average µg/g free residues[1]				Percent MS-222
	MS-222		m-aminobenzoic acid		
	Mean	Range	Mean	Range	
0	21.7	12.3-31.9	12.7	10.0-15.3	62.8
1	13.2	5.8-19.6	17.0	16.3-17.6	43.7
3	5.2	0.4- 8.2	20.7	19.7-22.5	20.1
6	0.3	0.0- 0.9	23.7	21.8-26.4	1.3
8	0.0	--	24.9	22.5-28.2	0.0

[1] Average of three fish, which were exposed for 30 minutes in a 100-mg/l aqueous solution of MS-222 at 20° C.

Table 6.--Average concentrations of benzocaine and p-aminobenzoic acid residues in muscle tissue of five striped bass, bluegill, and largemouth bass following deep anesthesia in a 63.216-mg/l aqueous solution of benzocaine buffered to pH 6.5 at 17.8° C

Species	Exposure time (minutes)	Withdrawal time (hours)	Free benzocaine residues (μg/g)		Free p-aminobenzoic acid residues (μg/g)	
			Mean ± $s_{\bar{x}}$ [1]	Range	Mean ± $s_{\bar{x}}$	Range
Striped bass[2]	0	0	0.0	--	0.3± 0.09	0.0-0.5
	15	0	37.9 ± 2.43	28.6-41.4	1.5 ± 0.46	0.8-3.3
		1	7.3 ± 0.99	3.4- 8.8	0.2 ± 0.07	0.0-0.4
		4	0.7 ± 0.21	0.1- 1.2	0.1 ± 0.04	0.0-0.2
		24	0.2 ± 0.07	0.0- 0.5	0.0	--
Bluegill[3]	15	1	1.9 ± 0.51	0.9- 3.8	0.0	--
Largemouth bass[4]	15	1	2.9 ± 0.57	2.0- 4.8	0.2± 0.00	--

[1] Standard error of the mean for five fish.
[2] Average length of 16.0 cm and average weight of 54.5 g.
[3] Average length of 18.0 cm and average weight of 154.0 g.
[4] Average length of 19.1 cm and average weight of 106.8 g.

Both the ester and acid residues decreased during storage in a refrigerator at 1.7° ± 0.2° C for 1 week (table 3). This decrease was due mainly to acetylation of the free amine. Residues of benzocaine and p-aminobenzoic acid were confirmed in all of the 0-hour withdrawal samples of striped bass by thin-layer chromatography.

DISCUSSION

Striped bass muscle tissue contains an esterase or a substance capable of cleaving the ester of MS-222. Muscle tissue of striped bass spiked with MS-222 and analyzed 1 week later contained both MS-222 and m-aminobenzoic acid. It is not known whether the muscle tissue is completely responsible for the ester cleavage while the fish is still alive. Preliminary investigations showed that m-aminobenzoic acid residues amounted to 27.1 percent of the total free residues in striped bass muscle tissue, 20.6 percent in blood, and 15.8 percent in liver after a 30-minute exposure in a 100-mg/l solution of MS-222. In the 1-hour withdrawal samples, muscle tissue had the lowest concentration of free acid when compared to blood and liver. No residues of acetylated MS-222 were detected in the 0-hour withdrawal samples of blood, liver, and muscle. In the 0-hour withdrawal samples, residues of m-acetylaminobenzoic acid were highest in the blood (22.5 μg/g) and lowest in the muscle (1.7 μg/g).

A limited amount of information is available on the acid metabolite of MS-222, m-aminobenzoic acid, in freshwater fishes. On the other hand, a considerable amount of data is available on the N-acetyl derivative of MS-222, but no differentiation is made between the acetylated ester and the acid (Schoettger, Walker, Marking, and Julin, 1967; Hunn, Schoettger, and Willford, 1968). Presumably MS-222 and acetylated MS-222 are eliminated by the gills during recovery, whereas m-aminobenzoic acid and m-acetylaminobenzoic acid are excreted renally (Hunn, 1970). Investigations by Maren, Broder, and Stenger (1968) on the metabolism of ethyl-m-aminobenzoate (MS-222) in the dogfish shark (Squalus acanthias), revealed that 2 hours after dogfish sharks were

injected with MS-222, the predominant metabolites in the urine were m-aminobenzoic acid and m-acetylaminobenzoic acid. Hunn, Schoettger, and Willford (1968) reported that acetylated MS-222 was found in much higher concentrations in the urine than in the blood of rainbow trout (Salmo gairdneri). No analyses were done on urine of striped bass.

In this study, residues of MS-222 and m-aminobenzoic acid steadily decreased with length of recovery time. On a percentage basis of all residues determined, m-aminobenzoic acid residues increased with recovery time in striped bass muscle tissue, but not in muscle tissue of bluegill and largemouth bass. Residue concentrations of m-aminobenzoic acid increased with an increase in sample storage time only in striped bass muscle tissue, whereas residue concentrations of p-aminobenzoic acid remained relatively low in fresh and stored muscle tissue of all fish analyzed. The decrease in the concentration of free benzocaine residues which occurred in all stored fish samples was due mainly to acetylation of the free amine. Thus, samples should be analyzed as soon as possible to obtain data pertinent to each withdrawal interval.

Residues of acetylated MS-222 (m-acetylaminobenzoic acid ethyl ester) cannot be determined accurately by the acetylation test of Walker and Schoettger (1967b). In this test the acetylated fraction is obtained by subtracting the free MS-222 and background concentrations from the total aromatic amines measured in the sample after acid hydrolysis. When the ester and acid fractions are separated before and after acid hydrolysis, the MS-222 fraction obtained after hydrolysis is generally less than the amount obtained before hydrolysis. This indicates that some free MS-222 is converted to free m-aminobenzoic acid during the acid hydrolysis. Thus, the esterified compounds should be quantified before and after acid hydrolysis, to determine the amount of free MS-222 and acetylated MS-222 that might be converted to free m-aminobenzoic acid during hydrolysis.

CONCLUSIONS

1. Among the species of fish analyzed to date, only the striped bass effectively hydrolyzes the ester of MS-222 to m-aminobenzoic acid in vivo.

2. Hydrolysis of MS-222 also occurs in frozen homogenized muscle tissue from treated striped bass.

3. A reduction in the concentration of MS-222 residues after 1 week storage at 1.7° C in bluegill and largemouth bass muscle tissue was due mainly to the acetylation of the free amine.

4. Analysis of striped bass muscle tissue for MS-222 residues must be done soon after the fish are killed to determine the amount of parent drug rather than its acid metabolite, because hydrolysis occurs during storage.

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U.S. GOVERNMENT PRINTING OFFICE : 1974 O—527-811

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